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Jones, C; Helfer, G; Brandstaetter, Roland

DOI:
10.3109/07420528.2011.642912

Publications

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Submitted to Chronobiology International in April 2011, Accepted in November 2011, due to be February 2012 edition.

Melatonin receptor expression in the zebra finch brain and peripheral tissues

Catherine Jones, Gisela Helfer, Roland Brandstaetter

Biological Rhythms Research Group, School of Biosciences, LES College, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK. E-Mail: r.brandstaetter@bham.ac.uk

Abstract

The circadian endocrine hormone melatonin plays a significant role in many physiological processes such as modulating sleep/wake cycle and oxidative stress. Melatonin is synthesised and secreted during the night by the pineal gland and released into the circulatory system. It binds to numerous membrane, cytosolic and nuclear receptors in the brain and peripheral organs. Three G-protein linked membrane receptors (Mel-1A, Mel-1B and Mel-1C) have been identified in numerous species. Considering the importance of this hormone and its receptors, this study looks at the location and rhythmicity of three avian melatonin receptors *Mel-1A*, *Mel-1B* and *Mel-1C* using reserve transcription-polymerase chain reaction (RT-PCR) mRNA analysis techniques. This study shows successful partial cloning of the three receptors and gene expression analysis revealed significant rhythms of the *Mel-1A* receptor in the cerebellum, diencephalon, tectum opticum, telencephalon, and retina. Significant rhythms where found in the diencephalon, pineal gland, retina, tectum opticum and cerebellum of the *Mel-1B* receptor whereas *Mel-1C* appeared not to be rhythmically expressed in brain tissues studied. *Mel-1A*, *Mel-1B* and *Mel-1C* receptor mRNA where also present in peripheral tissues showing tissue-specific expression patterns.

Keywords: brain; melatonin receptors; pineal gland; rhythm;

Address correspondence to Dr Roland Brandstaetter, Biological Rhythms Research Group, School of Biosciences, LES College, University of Birmingham, B15 2TT Birmingham, UK.
E-Mail: r.brandstaetter@bham.ac.uk

INTRODUCTION

Organisms time behavioural and physiological processes by synchronizing them with periodically changing environmental factors such as the light/dark cycle (Pittendrigh, 1993). In vertebrates, three central nervous structures have been identified to contain autonomous oscillators that are involved in the regulation of circadian rhythms: the retina, the pineal gland and the hypothalamus (Menaker et al., 1997). The relative contribution of these oscillators to the overall functioning of the circadian system at the whole-organism level varies among vertebrate species. In mammals, it is the hypothalamic oscillator that plays a major role in circadian organisation; it is located in a paired cell group in the hypothalamus, the suprachiasmatic nucleus (SCN) (van Esseveldt et al., 2000).

In birds, melatonin released from the pineal gland (e.g. songbirds) or from the retina and the pineal gland (e.g. galliform birds) appears to act as the major circadian coordinator and neural and physiological interactions of the retina, pineal gland, and hypothalamic oscillators determine circadian organisation at the whole-organism level (Gwinner and Brandstaetter, 2001; Brandstaetter, 2002; Brandstaetter, 2003). The two major input mechanisms to the hypothalamic circadian oscillators are light and melatonin; light can be perceived by photoreceptors found in the retina (mammals and birds), pineal gland (birds and lower invertebrates) and brain (birds and lower vertebrates) (Peirson et al., 2009). Pineal and retinal melatonin appear to inhibit activity in the hypothalamus during the dark phase, and the hypothalamus inhibits pineal melatonin production during the day (Karaganis et al., 2009).

Melatonin (5-methoxy-N-acetyltryptamine) is a small lipophilic hormone found in all living organisms from plants to humans (Carlberg, 2000) and has been found to be synthesized in both the retina (inner and outer retina) and the pineal gland (pinealocytes) of vertebrates. Early receptor studies revealed a wide distribution of 2-[^{125}I]iodomelatonin binding

throughout the chicken brain as compared to the rodent brain where binding was restricted to a few discrete areas (i.e. suprachiasmatic nucleus (SCN), periventricular nucleus (PVN) and median eminence (ME)) (Siuciak, et al., 1991). Melatonin binding in the chicken brain was most prominent in regions associated with the visual system, such as the visual SCN (vSCN), tectum opticum, and thalamofugal areas (Siuciak et al., 1991). In LD conditions, highest binding was found in the late afternoon (Zeitgeber time 10) and rhythmicity was shown to persist in constant conditions (Brooks and Cassone, 1992).

The biological effects of melatonin are produced through the activation of three melatonin receptor types, while other effects are due to its role as a pervasive and powerful antioxidant with a particular role in the protection of nuclear and mitochondrial DNA (Reiter et al. 2003; Rada and Wiechmann 2006; Dufourny et al., 2008). In 1994, the first membrane receptor was cloned from *Xenopus laevis* immortalized melanophores, which was sensitive to guanine nucleotides and its activation lead to an inhibition of adenylyl cyclase through a pertussis toxin-sensitive mechanism (Ebisawa et al., 1994). Since then, three melatonin receptor genes have been identified, melatonin receptor 1A (in mammals and non-mammalian vertebrates), 1B (in mammals and some non-mammalian vertebrates) and 1C (in non-mammalian vertebrates). All three receptors contain 7 hydrophobic transmembrane domains, and are linked to the guanine nucleotide binding proteins (G-protein-coupled) receptor superfamily (Ebisawa et al., 1994; Reppert, 1997). The main differences between the membrane receptor types are their kinetic and pharmacological properties for melatonin and its agonists: Mel-1A is a high affinity binding receptor, whereas the Mel-1B receptor has a low affinity for binding (Dubocovich 1995). Chicken Mel1A is 80% identical to human Mel1A (Reppert et al. 1995) and is the structural homolog of the mammalian MT1 receptor. Chicken Mel-1C receptor has an 80% homology to the Mel-1C found in Xenopus, and is 60% identical to the mammalian Mel-1A and -1B receptors (Reppert, 1997; Reppert et al. 1995). Mel-1C of lower vertebrates has an MT1-like pharmacology and receptor distribution suggests that melatonin may exert temporal control over a broad range of physiological and behavioural events in birds (Reppert et al., 1995; Sugden et al. 2004). Mel-1C has rapidly evolved from fishes and birds to mammals to the so-called GPR50 receptor that is believed to act as a modulator of melatonin responsiveness (Dufourny et al., 2008). There is a third transmembrane receptor (MT3) identified in mammals that is neither structurally nor physiologically comparable to the

receptors found in lower invertebrates; it has a 95% homology to human quinine reductase 2, a detoxification enzyme (Witt-Enderby et al., 2003).

Generally, removal of the pineal gland abolishes circadian rhythmicity of behaviour in songbirds highlighting the importance of circulating melatonin. Studies so far have looked at the removal of the whole gland (Gaston and Menaker, 1968), circulating plasma melatonin levels (Gwinner et al., 1997) and melatonin supplements (Heigl and Gwinner, 1995), but we still lack comprehensive information on the localisation and regulation of melatonin receptors in the brain of birds. The present study provides a first comprehensive investigation of melatonin receptor 1A (also referred to as Mel-1A, CKA MT1, MNRT1), 1B (also referred to as Mel1B, MT2, MTNR1B, ML1B) and 1C (also referred to as Mel1C, CKB) expression in the brain of a songbird, the zebra finch; we used the most frequently used non-mammalian nomenclature, i.e. Mel-1A, Mel-1B, and Mel-1C, in this paper as the present classification and nomenclature of melatonin receptors were applied only to mammalian receptors and there is no consensus on classifying nonmammalian receptors at present (Dubocovich et al., 2010).

MATERIALS AND METHODS

Animals and synchronisation

Adult male and female zebra finches were kept in light-tight and sound proof indoor aviaries with water and food *ad libitum* (Foreign finch diet seed, cuttlefish bone, mineralise grit and fresh fruit and vegetables). The aviaries were equipped with passive infrared sensors connected to a data acquisition computer system to record locomotor and feeding activities with CLOCKLAB software. The birds were kept in a light-dark cycle of 12hrs of bright light from 09:00h (ZT 0) to 21:00h (ZT12) and 12hrs of dim light (0.1lux) from 21:00h (ZT12) to 09:00h (ZT0) (LD12:12) for at least three weeks to ensure the birds where fully synchronised before tissue sampling. All experimental procedures were performed according to the UK home office regulations and international ethical standards (Portaluppi et al., 2010).

Tissue Sampling

Zebra finches were killed by dislocation of the neck and decapitation at six different time points over 24hrs (ZT 2, 6, 10, 14, 18, and 22). The brains (n = 6/ZT) were quickly removed from the skull; after the pineal gland was removed, the brain was dissected into the cerebellum, diencephalon, telencephalon, tectum opticum and the retina was sampled thereafter. For peripheral tissues, an incision was made along the chest bone and the rib cage was opened up and tissue samples of heart, liver, lungs and kidneys obtained. All tissues were immediately frozen on dry ice and then stored at -80°C until further use.

RNA extraction and cDNA synthesis

Total RNA was isolated by homogenising tissue samples with a ball mill (Mixer Mill MM 300, Retsch, Haan, Germany) following the protocol of Helfer et al. (2006). 3mm tungsten carbide beads were used to ensure full homogenisation of all tissue samples. The total RNA fractions from the samples were isolated using Trizol™ (Invitrogen Life Technologies, Carlsbad, USA). The resulting RNA samples were analysed by Nano-drop (Thermo Scientific, Wilmington, USA), Bioanalyzer RNA 6000 Nano assay (Agilent Technologies, Santa Clara, USA) and RNA gels. RNA samples were incubated with RNase-free DNase l (Roche Diagnostic, Mannheim, Germany) before 1.0µg aliquot of the resulting RNA was reverse transcribed using oligo(dT) primers. The product was then subjected to Reserve Transcription Polymerase Chain Reaction (RT-PCR) to amplify the cDNA content. Due to the small size of the avian pineal gland less than 1µg of total RNA was available for the first strand cDNA synthesis.

Amplification and Identification of zebra finch Mel-1A, Mel-1B and Mel -1C receptors cDNA

cDNA samples were taken from the diencephalon (DI) at time points ZT 6, 14 and 18. Primer pairs (Table. 1) were added to the relevant cDNA samples and amplified with RT-PCR using the Taq DNA Polymerase kit (Roche Diagnostics; thermocycler, Applied Bioscience, Gene Amp PCR system 9700) under the following conditions: 94^{o}C/2 min, 1 cycle; 94^{o}C/30seconds, 55^{o}C/30seconds, 72^{o}C/1min, 10 cycles; 94^{o}C/30seconds, 60^{o}C/30seconds,

72°C/1min (+ 5 sec to each subsequent run), 30 cycles; 72°C/7 min, 1 cycle; 4°C/hold. In every PCR reaction negative controls, i.e. no cDNA, were included to control for contamination. PCR products (n = 2/ZT 6, 14, 18) were separated on a 2% agarose gel and visualised on a UV light plate and dissected out. The DNA was extracted from the gel using gel extraction kit (QIAGEN, QIAquick gel extraction kit). The DNA sample from each band were ligated into a T-tail cloning vector, using pGEM-T easy vectors (Promega, Madison, USA). The plasmid vectors with the DNA insert were purified from transformed competent DH5α cell colonies by alkaline lysis (High pure plasmid isolation, Roche Diagnostics) and digested with EcORI (37°C for 55min). The relevant PCR products were sequenced, analysed and aligned using Chromas (Technelysium Pty Ltd), BioEdit (Ibis Biosciencs, Carlsbad, CA), NCBI Blast database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and Clustal W2 program (http://www.ebi.ac.uk/Tools/clustalw2).

Optimising RT-PCR conditions for Mel-1A, Mel-1B and Mel-1C receptors

PCR conditions were optimised to obtain amplified specific receptor PCR products in the linear phase of synthesis in three steps; Step 1: magnesium concentrations (0-2.5mM) and annealing temperatures (55°C, 57°C, 60°C, 62°C), Step 2: cycle numbers (10-42 cycles, every 2 cycle numbers were tested), and Step 3: cDNA dilution series . Optimised values for each gene primer pair are shown in Table 2.

Semi-quantitative Reserve Transcription-PCR (RT-PCR) for Melatonin receptors 1A, 1B and 1C

The PCR conditions were 94°C/2 min, 1 cycle; 94°C/30seconds, 55°C/30seconds, 72°C/1min, 1 cycles; 94°C/30seconds, X°C/1min, 72°C/1min (5 sec increments in each subsequent run), Y cycles; 72°C/7 min, 1 cycle; 4°C/hold (optimised X and Y values see Table 2). PCR products were run on an 8% polyacrylamide gel electrophoresis and stained with SYBR green I (Roche Diagnostics). The amplified products were quantified with a Gel Doc Imaging System in combination with Quantity One imaging analysing software (BioRad, Hercules,

USA). To correct for gel staining variation, the optical densities of each sample were compared to a 50bp DNA ladder (Fermentas) with known marker concentration.

The resulting data for each gene/time-point/tissue were normalised to TATA-box binding protein (TBP) values for the same tissue as previously described (Helfer et. al, 2006). Melatonin receptor gene expression was analysed by one-way factorial analysis of variance (ANOVA) using Tukey's honest significant difference (HSD) post hoc test. Polynomial fourth order non-linear regression and Lowess curves were fitted with GraphPad Prism software (GraphPad, San Diego, CA, USA) to compare expression patterns as previously described (Brandstaetter et al., 2000).

Declaration of Interest

The authors report no conflicts of interest.

RESULTS

Amplification and Identification of zebra finch Mel-1A, Mel-1B and Mel-1C receptors cDNA

Using RT-PCR, we successfully cloned and sequenced partial cDNA for melatonin receptors 1A (494bp), 1B (265bp) and 1C (463bp) in zebra finch brain tissues. The sequences were compared with other avian DNA sequences using NCBI blast database and analysed accordingly showing 96% to 99% nucleotide sequence identities to previously published avian melatonin receptor sequences (Table 3).

Semi-quantitative RT-PCR for Melatonin receptors 1A, 1B and 1C in the zebra finch brain

Mel1A mRNA showed robust significant rhythmicity in two of the circadian oscillator regions, i.e. diencephalon and retina, with peak expression levels between ZT18 and ZT22 (ANOVA, $F_{5,27}$=8.210, p=0.0002 for diencephalon, $F_{5,25}$=5.836, p=0.0018 for retina). No

clear rhythmicity could be detected in the pineal gland with *Mel-1A* mRNA levels being low near the detection limit throughout light and dark phases (Fig. 1). *Mel-1B* mRNA was rhythmically expressed in all three of the oscillator regions with peak expression times of ZT22 to ZT2 in the diencephalon (ANOVA, $F_{5,20}$=7.010, p=0.0015), ZT22 in the pineal gland (ANOVA, $F_{5,19}$=4.233, p=0.0149), and ZT10 in the retina (ANOVA, $F_{5,24}$=4.446, p=0.0075) (Fig. 2). *Mel-1C* mRNA was present in all three oscillatory regions but did not show any rhythmicity (Fig. 3).

In the tectum opticum, *Mel-1A* mRNA was rhythmically expressed with low levels during the day and high levels during the dark phase with significant peak expression from ZT 18 to ZT 22 (ANOVA, $F_{5,31}$=8.944, p<0.0001) comparable to the cerebellum (ANOVA, $F_{5,32}$=3.170, p=0.0223) and the telencephalon (ANOVA, $F_{5,34}$=3.981, p=0.0072) (Fig. 1).

In the tectum opticum (ANOVA, $F_{5,20}$=5.752, p=0.004), cerebellum (ANOVA, $F_{5,16}$=5.079, p=0.001), and telencephalon, *Mel-1B* mRNA showed biphasic patterns of rhythmic expression with peak expression levels during the light phase as well as during the dark phase. While peak expression levels for *Mel-1B* mRNA were at ZT2 and ZT14 in the tectum opticum, i.e. two hours after the onset of light and two hours after the onset of darkness, peak expression levels in the cerebellum were reached at ZT2 and ZT 18 (Fig. 2). A similar pattern was observed in the telencephalon where expression values were found not to be significantly rhythmic.

Intermediate to high absolute *Mel-1C* mRNA levels could be found throughout the brain but without any clear signs of rhythmicity in any of the parts of the brain that were studied (Fig. 3). Absolute mRNA levels were comparable in all parts of the brain for *Mel-1A* while peak levels of *Mel-1B* where about four times higher in the retina as compared to all other parts of the brain.

Semi-quantitative RT-PCR for Melatonin receptors 1A, 1B and 1C in zebra finch peripheral tissues

To reveal whether similar patterns of melatonin receptor expression were detectable in peripheral tissues, melatonin receptor expression was also analysed in the heart, liver, lung

and kidney of the zebra finch. *Mel-1A* receptor mRNA levels in heart, lung and kidney were low and arrhythmic while there was a significant rhythm in the liver with peak expression at ZT2 (ANOVA, $F_{5,35}$=4.321, p=0.0044), two hours after the onset of light (Fig. 4).

Interestingly, three out of the four studied peripheral tissues showed significant *Mel-1B* receptor rhythms with tissue-specific peak expression times. In the heart, expression levels were high throughout the light period with peak expression at ZT2 and ZT10 (ANOVA, $F_{5,24}$=7.582, p=0.0005). Similarly, liver *Mel-1B* mRNA was significantly elevated throughout the light phase as compared to darkness (ANOVA, $F_{5,25}$=12.32, p<0.0001). In variance to heart and liver, lung *Mel1B* mRNA showed peak expression times at ZT10 and ZT12, i.e. at the transition from light to dark (ANOVA, $F_{5,27}$=8.591, p=0.0001). Although *Mel-1B* levels in the kidney were variable during the 24-hr cycle studied, no significant rhythm could be detected (Fig. 5).

Mel-1C receptor expression showed no apparent rhythmicity in either heart, liver or lung while there was a significant diurnal rhythm found in the kidney with peak expression levels at ZT2 (ANOVA, $F_{5,15}$= 5.449, p=0.01) (Fig. 6).

Absolute mRNA levels indicate clear differences in melatonin receptor density between tissues with *Mel-1A* being generally low in all peripheral tissues, *Mel-1B* showing high mRNA levels in heart, lung, and liver, and *Mel-1C* being highest in liver and kidney.

DISCUSSION

So far, melatonin studies in birds have mostly concentrated on plasma melatonin levels and behavioural effects of exogenous melatonin on physiology and behaviour. Studies on avian melatonin receptors mainly focused on the retina (Rada and Wiechmann, 2006), gonads (Aste et al., 2001) and song-control nuclei in the brain (Cassone et al., 2008; Whitfield-Rucker and Cassone, 1996) while localisation of melatonin receptors in the brain and rhythmicity of expression received very little attention. We cloned three melatonin membrane receptors in the zebra finch, *Mel-1A, Mel-1B* and *Mel-1C*, that show amino acid sequence homologies of 98-99% to previously published zebra finch melatonin receptors (Jansen et al., 2005); we found all of these melatonin receptor types to be expressed in all major divisions of the brain

as well as in all peripheral tissues studied. This is in remarkable contrast to the very restricted distribution of melatonin receptors in the mammalian brain. As compared to all other vertebrate classes, mammals have lost photosensitivity of the pineal gland as well as the circadian clock in the pineal gland, and have one dominant circadian brain oscillator, i.e. the suprachiasmatic nucleus, that controls pineal synthesis and secretion of nocturnal melatonin; photoreception takes place in the retina only and there has been an evolutionary loss of two retinal cone classes (Barrett et al., 2003) and of certain opsins which are found in non-mammalian vertebrates including birds and fishes (Bellingham et al., 2003). These differences in circadian organisation are believed to be the consequence of a "nocturnal bottleneck" during early evolution of mammals (Foster et al., 1993). The striking difference in melatonin receptor distribution between birds and mammals is another sign of the different evolutionary circadian paths of mammals as compared to non-mammalian vertebrates. The ubiquituous presence of all melatonin receptor types throughout the brain and in all peripheral tissues of the zebra finch supports the hypothesis that melatonin acts as the major coordinator of circadian organisation at the whole-organism level in non-mammalian vertebrates such as birds. Non-mammalian vertebrates, including birds, possess complex circadian systems with multiple photic input mechanisms and multi-oscillator control of circadian rhythmicity with melatonin being believed to act as the major driver of coordinated circadian rhythmicity at the whole-organism level (Gwinner & Brandstaetter, 2001). We found *Mel-1A*, *Mel-1B* and *Mel-1C* receptor mRNA expression in all parts of the avian brain that have been shown to contain autonomous circadian clocks, i.e. retina, pineal gland and diencephalon. *Mel-1A* and *Mel-1B* were found to be rhythmic in the retina and diencephalon while although *Mel-1C* was found in these tissues its expression was not rhythmic. *Mel-1B* was found to be rhythmic in the pineal gland in contrast to *Mel-1A* and *Mel-1C* mRNA. *Mel-1A* mRNA was found to show nocturnal rhythms with peak expression levels during the second half of the night in all parts of the brain apart from the pineal gland. *Mel-1B* receptor expression was most variable with nocturnal rhythms in the pineal gland and the diencephalon reaching highest levels at the end of the night and the transition from dark to light, a diurnal rhythm in the retina, and biphasic expression patterns in the tectum opticum, cerebellum, and telencephalon. *Me-l1C* receptor was not found to be rhythmic in any of the brain regions studied although considerable mRNA levels were present. These results show the widespread distribution of all melatonin

receptors in the avian brain and suggest tissue-specific regulation of melatonin receptor expression.

The non-rhythmic but considerable expression of *Mel-1C* in all tissues studied is another remarkable outcome of this study. To exert full physiological action, melatonin receptors may form functional dimers (Ayoub et al., 2002) and it has been shown that the mammalian GPR50 receptor modulates the function of mammalian MT1 and MT2 receptors (Levoye et al. 2006). Our results showing the ubiquitous presence of all three receptors throughout the brain and in all peripheral tissues studied suggests that the modulatory role of Mel-1C/GPR50 found in mammals may also be important in birds with Mel1C possibly being the partner of the Mel1A/Mel1B receptor heterodimers. Rhythmic expression of either *Mel-1A* or *Mel-1B* or both was indeed what we found in our study in all parts of the brain and in all peripheral organs. Strikingly, the only tissue where neither *Mel-1A* nor *Mel-1B* mRNA was rhythmically expressed was the kidney where rhythmic *Mel-1C* receptor expression was found instead.

In passerine birds, such as the zebra finch and house sparrow, the only source of circulating melatonin is the pineal gland (Brandstaetter, 2002). As melatonin production is controlled by an autonomous circadian clock in songbirds, one would predict that there would be no melatonin membrane receptors found in this gland unless they serve auto-regulatory feedback. Our data set confirms that all three melatonin receptors are present in the pineal gland with *Mel-1B* being the only receptor that was rhythmically expressed. Interestingly, pineal *Mel-1B* receptor expression appears to peak at the end of the dark phase (ZT 22) when melatonin production declines suggesting that *Mel-1B* receptors may indeed act as negative feedback and contribute to the termination of melatonin production at the end of the night.

Interestingly, melatonin receptor expression mirrors nocturnal circulating melatonin in some but not all tissues; while *Mel-1A* receptor expression reflects the presence of nocturnal melatonin in the diencephalon, retina, tectum opticum, cerebellum, and telencephalon with peak expression levels during the second half of the night, *Mel-1B* expression shows considerable variability between brain regions, and *Mel-1C* does not appear to be rhythmically expressed at all. The overall differences in absolute melatonin receptor mRNA levels are comparable with the findings of Reppert et al. (1995) in the chicken where no Mel1A was found within the cerebellum, liver, or pineal gland but within the diencephalon, telencephalon, retina and tectum opticum while high concentration of *Mel-1B* were detected

in the tectum opticum, hypothalamus, thalamus and pineal gland and low *Mel-1B* concentration were found in the cerebellum and retina but not in the liver (Reppert et al., 1995).

Rada and Wiechmann (2006) identified all three melatonin receptor types in the chicken retina-RPE-choroid and found Mel-1A and Mel-1B to have similar expression patterns with low levels occurring in the early morning and highest levels in the evening (Mel-1A peaked at ZT12 (lights off) and Mel-1B peaked at ZT20 (mid-late dark period) while Mel-1C showed out-of-phase oscillation to Mel-1A and Mel-1B with high levels in the early morning and low levels during the early-mid dark phase (ZT16). Chicken retinal *Mel-1A* mRNA has been found to be rhythmic under light/dark (LD) cycles and in constant darkness (DD), peaking at midday and mid-subjective day (Natesan and Cassone 2002). Chicken retinal *Mel-1C* mRNA was also found to be rhythmic in LD with high expression levels during the day but opposite phasing in DD while *Mel-1B* was highly variable and arrhythmic (Natesan and Cassone, 2002). A comparison of our mRNA data with melatonin receptor protein levels in the chicken retina revealed certain differences in peak expression times; while the *Mel-1A* receptor was found to peak at ZT12 at the transition from light to dark, *Mel-1B* peaked at ZT20, and *Mel-1C* at ZT0 in the chicken retina (Rada & Wiechmann, 2006) we found mRNA in the retina to peak at ZT18 (*Mel-1A*), ZT10 (*Mel1B*), and *Mel1C* mRNA to be arrhythmic. These differences could be caused by differential expression of melatonin receptors in the different layers of the retina as both studies used whole tissue samples or by species-specific differences between the role the retina plays in galliform and passeriform circadian organisation (Brandstaetter, 2002).

Generally, the actions of melatonin at the cellular level are very complex due to the presence of several receptor types with different affinities for melatonin, due to complex interaction mechanisms between the receptors, and due to possible melatonin action via membrane receptors but also nuclear and cytosolic binding (Reiter et al. 2003; Levoye et al., 2006; Rada and Wiechmann 2006). In mammals, the MT1 receptor has been implicated in inhibiting neuronal firing within the SCN (Dubocovich, 2007) possibly due to the inhibitory responses on the cAMP signal transduction cascade by decreasing PKA activity and CREB phosphorylation (Witt-Enderby et al., 2003). MT1 has also been shown to inhibit the induction of c-fos and junB mRNA and c-fos translation (Witt-Enderby et al., 2003).

Circadian phase shifting of the neuronal firing rhythms, however, has been linked to the activation of MT2 receptor (Dubocovich, 2007) and recent findings indicate that melatonin mainly acts by altering neuronal excitability in SCN neurons by modulating inhibitory GABAergic transmission within the SCN (Scott et al., 2010). In contrast to mammals, very little is known about the physiological cellular effects of melatonin in birds but there is clear evidence that the pineal melatonin rhythm acts on at least one other oscillator within the circadian pacemaking system, presumably the hypothalamic clock, which in turn feeds back onto the pineal gland (Gwinner and Brandstaetter, 2001).

In variance to melatonin receptor expression in the brain of the zebra finch, peripheral tissues showed considerable variability; while liver showed rhythmic *Mel1A* and *Mel1B* expression, only *Mel1B* receptor mRNA was found to be rhythmically expressed in lung and heart. Interestingly, kidney, as the only tissue in this study, showed a *Mel1C* receptor rhythm which is of particular interest regarding its possible role as a modulator of melatonin receptor function (Levoye et al., 2006). Peripheral tissues also differed in regard of the temporal organisation of melatonin receptor expression. While *Mel1A* in the liver and *Mel1C* in the heart peak early in the day when circulating melatonin has already declined to baseline levels (Brandstaetter et al., 2001), *Mel1B* is elevated throughout the day in heart and liver but peaks at the transition from light to dark in the lung. These data suggest complex control mechanisms of melatonin receptor expression in peripheral organs and diurnal rhythms of melatonin responsiveness that may relate to the rhythm of circulating melatonin (Gwinner & Brandstaetter, 2001; Van't Hof & Gwinner, 1999).

In summary, our results represent a first comprehensive analysis of all three melatonin receptors at the mRNA level in a songbird species, the zebra finch. Although we cannot conclude on the physiological effects of melatonin nor on the dynamics of melatonin receptors at the protein level at present, we show brain region-specific and peripheral tissue-specific rhythms of two of the melatonin receptors *Mel-1A* and *Mel-1B* while *Mel-1C* receptor was present throughout the brain and in all peripheral tissues studied but no rhythmic expression pattern was found apart from one peripheral tissue, the kidney. Our results show very clearly that melatonin, in strong contrast to mammals, has the general ability to exert its actions throughout the brain and body in birds via all three receptor types. The differences in temporal regulation and overall receptor levels as indicated by our mRNA data set suggest

tissue-specific melatonin action in the different parts of the brain and in peripheral organs. The presence of each of the three receptor types in every tissue studied suggests widespread melatonin responsiveness throughout the brain and body of birds and has to be considered in future physiological and behavioural experiments to elucidate the multiple roles melatonin may play in co-ordinating circadian organisation at the whole-organism level in birds.

ACKNOWLEDGEMENTS

Financial support was provided by the Max Planck Society and the Biotechnology and Biological Sciences Research Council. We gratefully acknowledge information on melatonin receptor sequence data provided by M. Gahr (Max-Planck-Institute of Ornithology).

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Table 1. Primer sequences and predicted product length used to clone melatonin receptors Mel-1A, Mel-1B and Mel-1C from zebra finch cDNA templates. The Mel-1B primers are based on the published zebra finch Mel-1b receptor sequence (NM_001048258). The Mel-1A

and Mel-1C primers are based on sequences published on house sparrow (Mel-1A - AY155489 and Mel-1C - AY743658).

Table 2. Primer sequences and optimised PCR conditions for the Mel-1A, Mel-1B and Mel-1C partial cDNA gene homologues.

Table 3. Percentage nucleotide sequence identity of cloned Melatonin receptors Mel-1A, Mel-1B and Mel-1C from Zebra Finch diencephalon tissue cDNA at time points ZT=14 (CLON 2.1), ZT=6 (CLON 4.2) and ZT=14 (CLON 5.2).

Figure 1. Expression profiles of *Melatonin receptor 1A* mRNA of zebra finch brain tissues. Temporal expression of normalised data (A-F), i.e. relative mRNA values normalised to *TBP* mRNA expression shown as variation from mean value, and (G-L) absolute mRNA values, i.e. raw data. The bars at the top of each graph indicate the LD 12:12 hrs light/dark schedule the birds were exposed to. The symbols represent mean values for each time point ± SEM (n=4-6). ZT2 and ZT22 are double plotted for better visualisation. Fourth order polynomial (dashed line) and lowess (black line) curves were fitted with GraphPad prism. Significant differences as revealed by ANOVA indicated by * on figure letter; significant differences between times points, i.e. to the lowest value of mRNA expression, as revealed by post hoc Tukey test indicated by * above the corresponding symbols: *$p<0.05$, **$p<0.01$, ***$p<0.001$. X-axis: Zeitgeber time in hrs.

Figure 2. Expression profiles of Melatonin receptor 1B mRNA of zebra finch brain tissues. Temporal expression of normalised data (A-F), i.e. relative mRNA values normalised to *TBP* mRNA expression shown as variation from mean value, and (G-L) absolute mRNA values, i.e. raw data. The bars at the top of each graph indicate the LD 12:12 hrs light/dark schedule the birds were exposed to. The symbols represent mean values for each time point ± SEM (n=4-6). ZT2 and ZT22 are double plotted for better visualisation. Fourth order polynomial (dashed line) and lowess (black line) curves were fitted with GraphPad prism. Significant differences as revealed by ANOVA indicated by * on figure letter; significant differences between times points, i.e. to the lowest value of mRNA expression, as revealed by post hoc Tukey test indicated by * above the corresponding symbols: *$p<0.05$, **$p<0.01$, ***$p<0.001$. X-axis: Zeitgeber time in hrs.

Figure 3. Expression profiles of Melatonin receptor 1C mRNA of zebra finch brain tissues. Temporal expression of normalised data (A-F), i.e. relative mRNA values normalised to *TBP* mRNA expression shown as variation from mean value, and (G-L) absolute mRNA values, i.e. raw data. The bars at the top of each graph indicate the LD 12:12 hrs light/dark schedule the birds were exposed to. The symbols represent mean values for each time point ± SEM

(n=4-6). ZT2 and ZT22 are double plotted for better visualisation. Fourth order polynomial (dashed line) and lowess (black line) curves were fitted with GraphPad prism. Significant differences as revealed by ANOVA indicated by * on figure letter; significant differences between times points, i.e. to the lowest value of mRNA expression, as revealed by post hoc Tukey test indicated by * above the corresponding symbols: *p<0.05, **p<0.01, ***p<0.001. X-axis: Zeitgeber time in hrs.

Figure 4. Expression profiles of Melatonin receptor 1A mRNA of zebra finch peripheral tissues. Temporal expression of normalised data (A-D), i.e. relative mRNA values normalised to *TBP* mRNA expression shown as variation from mean value, and (E-H) absolute mRNA values, i.e. raw data. The bars at the top of each graph indicate the LD 12:12 hrs light/dark schedule the birds were exposed to. The symbols represent mean values for each time point ± SEM (n=4-6). ZT2 and ZT22 are double plotted for better visualisation. Fourth order polynomial (dashed line) and lowess (black line) curves were fitted with GraphPad prism. Significant differences as revealed by ANOVA indicated by * on figure letter; significant differences between times points, i.e. to the lowest value of mRNA expression, as revealed by post hoc Tukey test indicated by * above the corresponding symbols: *p<0.05, **p<0.01, ***p<0.001. X-axis: Zeitgeber time in hrs.

Figure 5. Expression profiles of Melatonin receptor 1B mRNA of zebra finch peripheral tissues. Temporal expression of normalised data (A-D), i.e. relative mRNA values normalised to *TBP* mRNA expression shown as variation from mean value, and (E-H) absolute mRNA values, i.e. raw data. The bars at the top of each graph indicate the LD 12:12 hrs light/dark schedule the birds were exposed to. The symbols represent mean values for each time point ± SEM (n=4-6). ZT2 and ZT22 are double plotted for better visualisation. Fourth order polynomial (dashed line) and lowess (black line) curves were fitted with GraphPad prism. Significant differences as revealed by ANOVA indicated by * on figure letter; significant differences between times points, i.e. to the lowest value of mRNA expression, as revealed by post hoc Tukey test indicated by * above the corresponding symbols: *p<0.05, **p<0.01, ***p<0.001. X-axis: Zeitgeber time in hrs.

Figure 6. Expression profiles of Melatonin receptor 1C mRNA of zebra finch peripheral tissues. Temporal expression of normalised data (A-D), i.e. relative mRNA values normalised to *TBP* mRNA expression shown as variation from mean value, and (E-H) absolute mRNA values, i.e. raw data. The bars at the top of each graph indicate the LD 12:12 hrs light/dark schedule the birds were exposed to. The symbols represent mean values for each time point ± SEM (n=4-6). ZT2 and ZT22 are double plotted for better visualisation.

Fourth order polynomial (dashed line) and lowess (black line) curves were fitted with GraphPad prism. Significant differences as revealed by ANOVA indicated by * on figure letter; significant differences between times points, i.e. to the lowest value of mRNA expression, as revealed by post hoc Tukey test indicated by * above the corresponding symbols: *p<0.05, **p<0.01, ***p<0.001. X-axis: Zeitgeber time in hrs.

Table 1

Gene	**Primer Pair**	**Predicted Product length**
Mel-1A	Forward: 5'- TGCCACAG(C/T)CTCA(A/G)(A/G)TA(C/T)GAC-3' Reverse: 5'- AT(T/C/G)GC(A/G)ATT(A/G)AGGCAGCTGTTGA-3'	500bp
Mel-1B	Forward: 5'-GACAAAGTGTACAGCTGTTGG-3' Reverse: 5'-CTGATTTGACTCGTCTTCGAAC-3'	265bp
Mel-1C	Forward: 5'- TGCT(A/G)CATCTGCCACAGCCT-3' Reserve: 5'-(GCT(C/T)A(G/A)(G/A)ACAAA(A/C)AGCCA(T/C)TCTG-3'	463bp

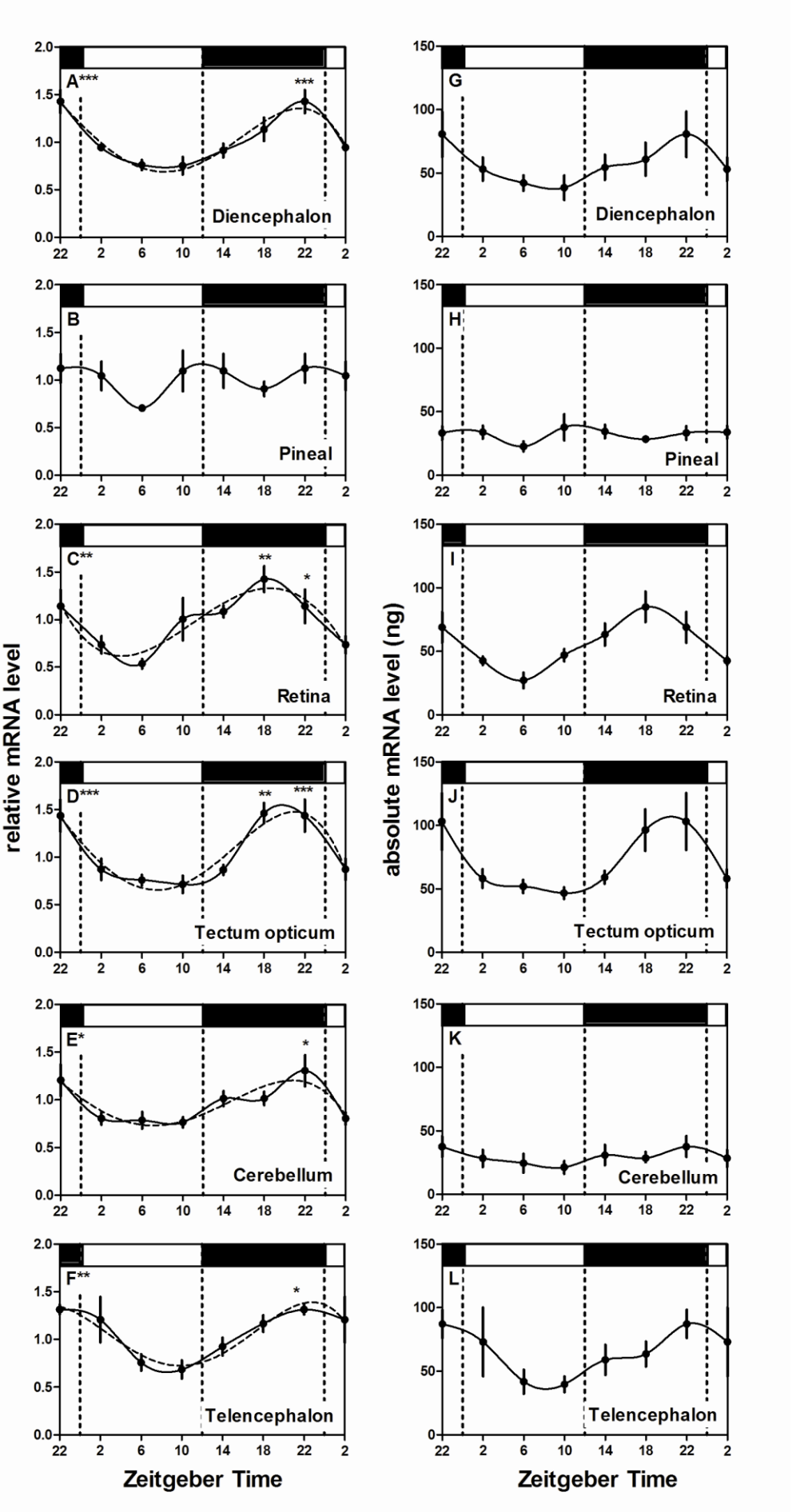
Table 2

Gene	RT-PCR Primer	Product length	Tm (X°C)	Cycle No. (Y)	Mg^{2+} Conc
Mel-1A	Forward: 5'-CCACAGTCTCAGATACGACAAGC-3' Reserve: 5'-ACCCTTCGCCTTACCTGGATAAC-3'	276	62°C	26	2mM
Mel-1B	Forward: 5'-GACAAAGTGTACAGCTGTTGG-3' Reserve: 5'-CTGATTTGACTCGTCTTCGAAC-3'	265	60°C	27	2mM
Mel-1C	Forward: 5'-TCTGCCTGACCTGGATACTCAC-3' Reserve: 5'- CTGCTTGCAGTCTTGTCTCACC-3'	239	62°C	27	1.5mM

Table 3

Cloned DNA sequence	Melatonin Receptor (GenBank accession number)	Species	Percentage Identity
Mel-1A Receptor	Mel-1A receptor mRNA; complete cds (NM_001048257.1)	Taeniopygia guttata (Zebra Finch)	98%
	Mel-1A receptor mRNA; partial cds (AY155489.1)	Passer domesticus (House Sparrow)	97%
	Mel-1A melatonin receptor mRNA, complete cds (GGU31820)	Gallus gallus (Chicken)	89%
	Melatonin receptor 1A (MTNR1A) mRNA, complete cds (EU432127.1)	Homo sapien (human)	76%
Mel-1B Receptor	Mel-1b melatonin receptor mRNA, complete cds (DQ178665)	Taeniopygia guttata (Zebra Finch)	99%

	Mel-1b melatonin receptor mRNA, partial cds (DQ178663.1)	Sylvia atricapilla (Blackcap)	96%
	Melatonin receptor 1b mRNA, partial cds (EF197909.1)	Gallus gallus (Chicken)	90%
	Melatonin 1b receptor, complete cds (AB033598.2)	Homo sapien (Human)	74%
Mel-1C Receptor	Mel-1C receptor mRNA; partial cds (AY803773.1)	Taeniopygia guttata (Zebra Finch)	99%
	Mel-1C receptor mRNA; partial cds (AY743658.1)	Passer domesticus (House Sparrow)	98%
	Mel-1C melatonin receptor mRNA, complete cds (U31821.1)	Gallus gallus (Chicken)	92%
	G protein-coupled receptor 50 (GPR50), mRNA (NM_004224)	Homo sapien (Human)	67%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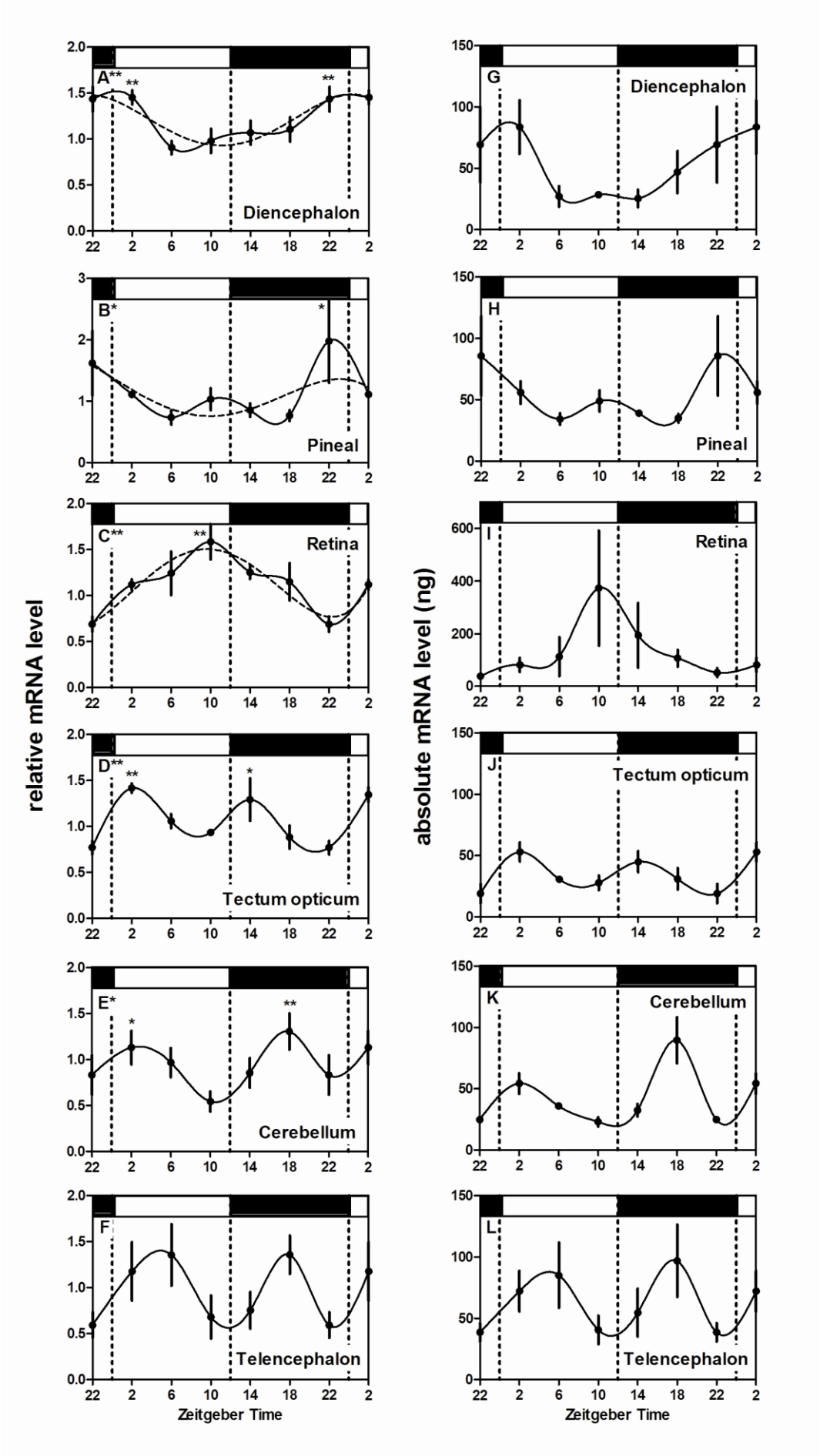
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Cerebellum
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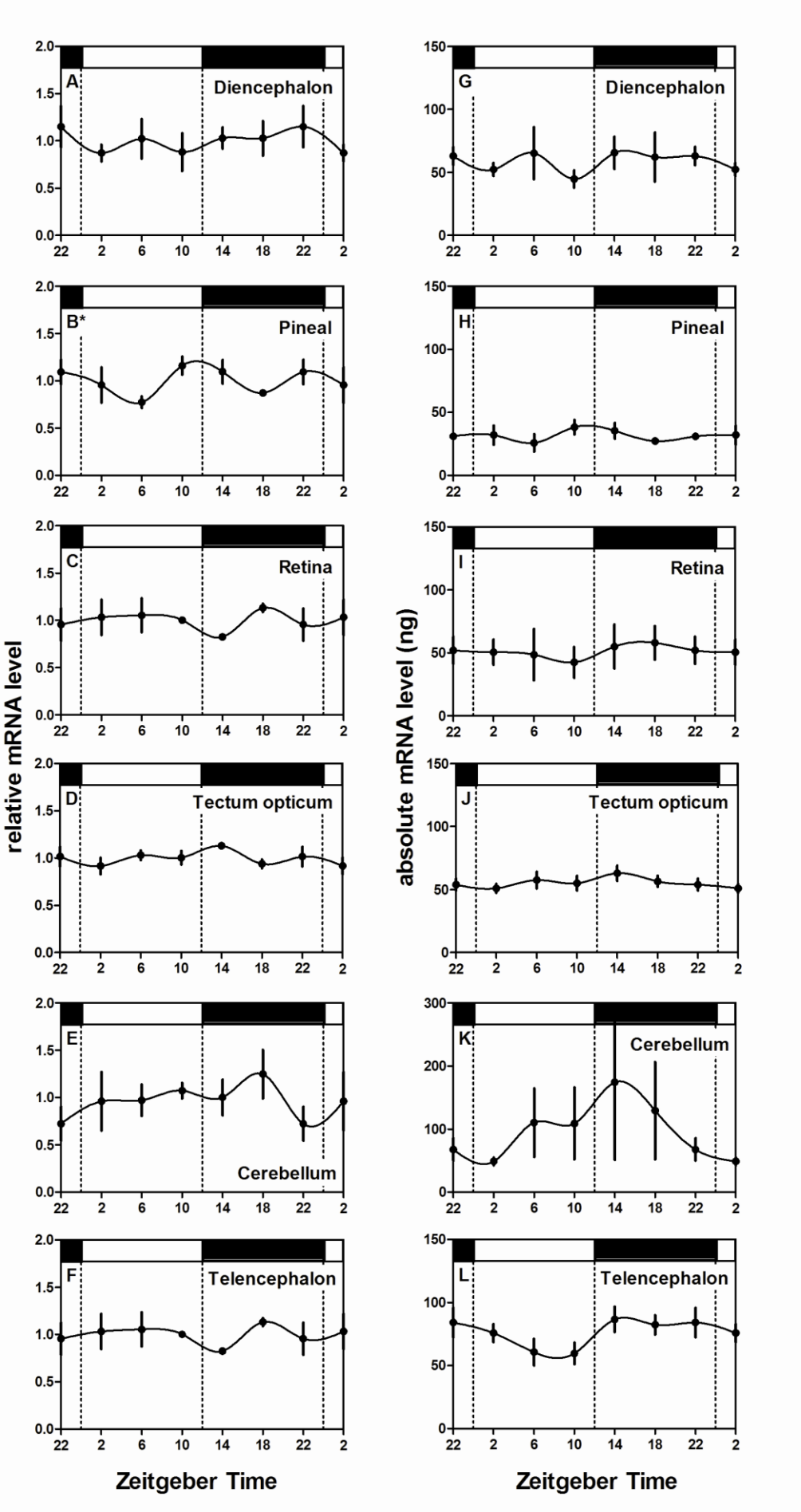
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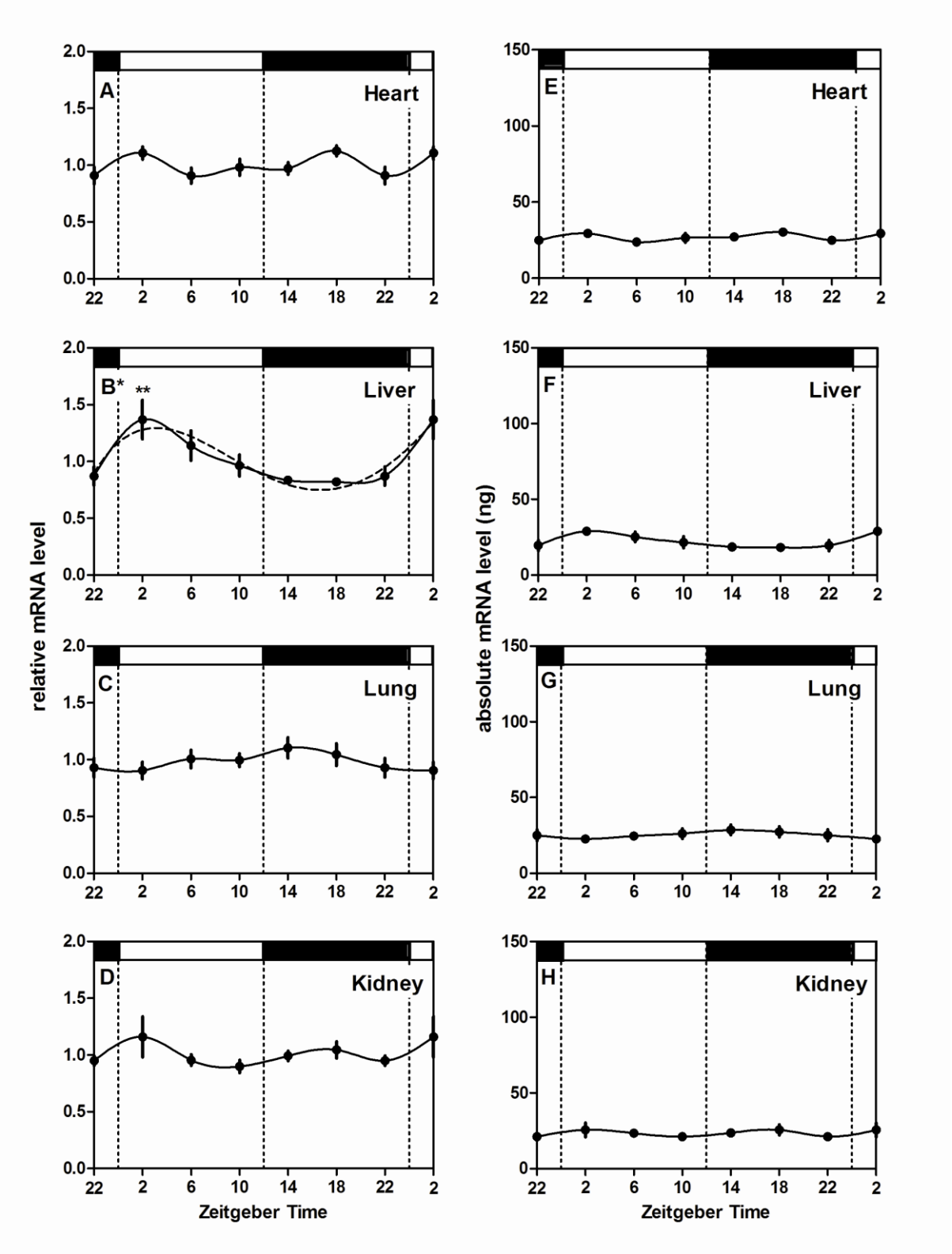
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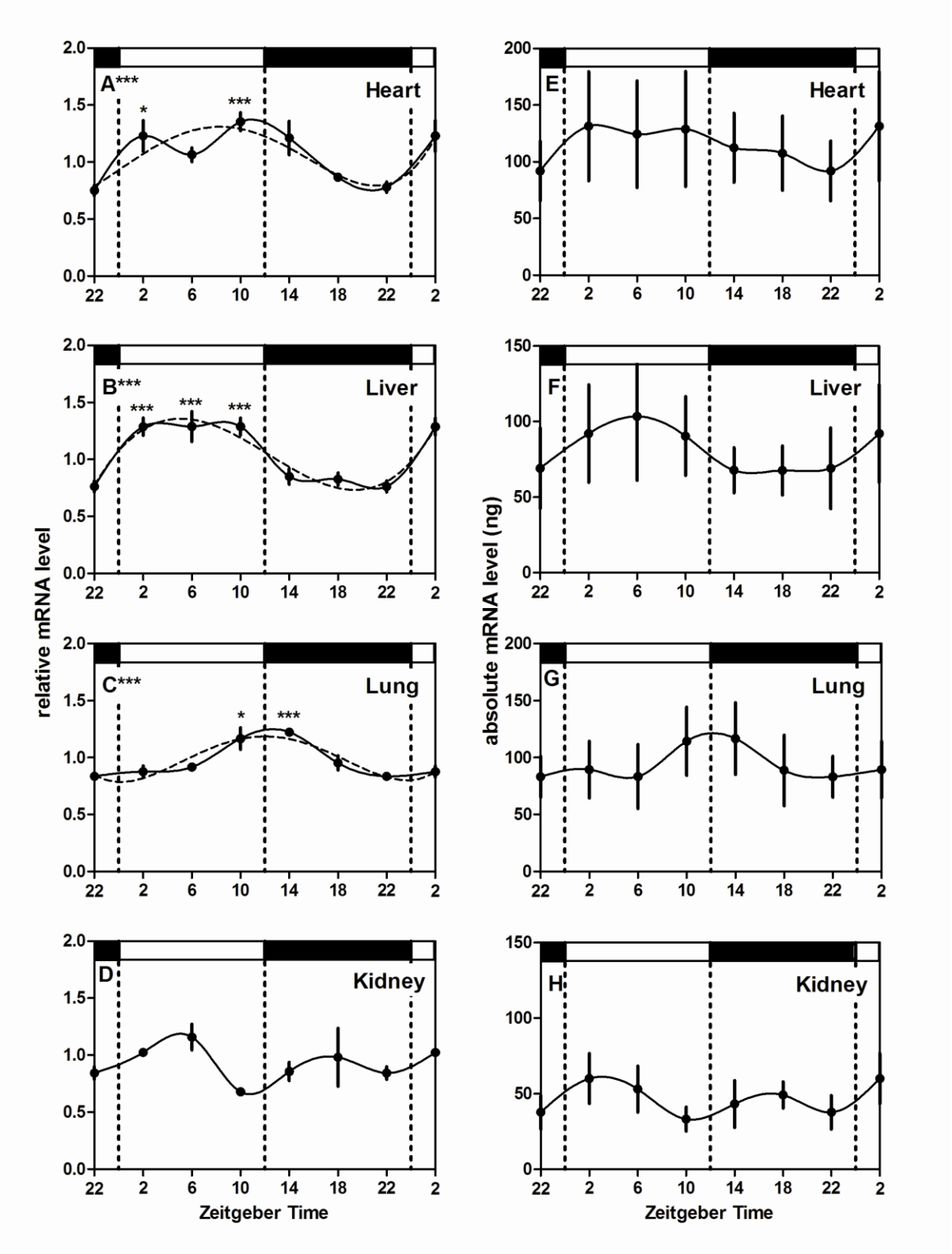
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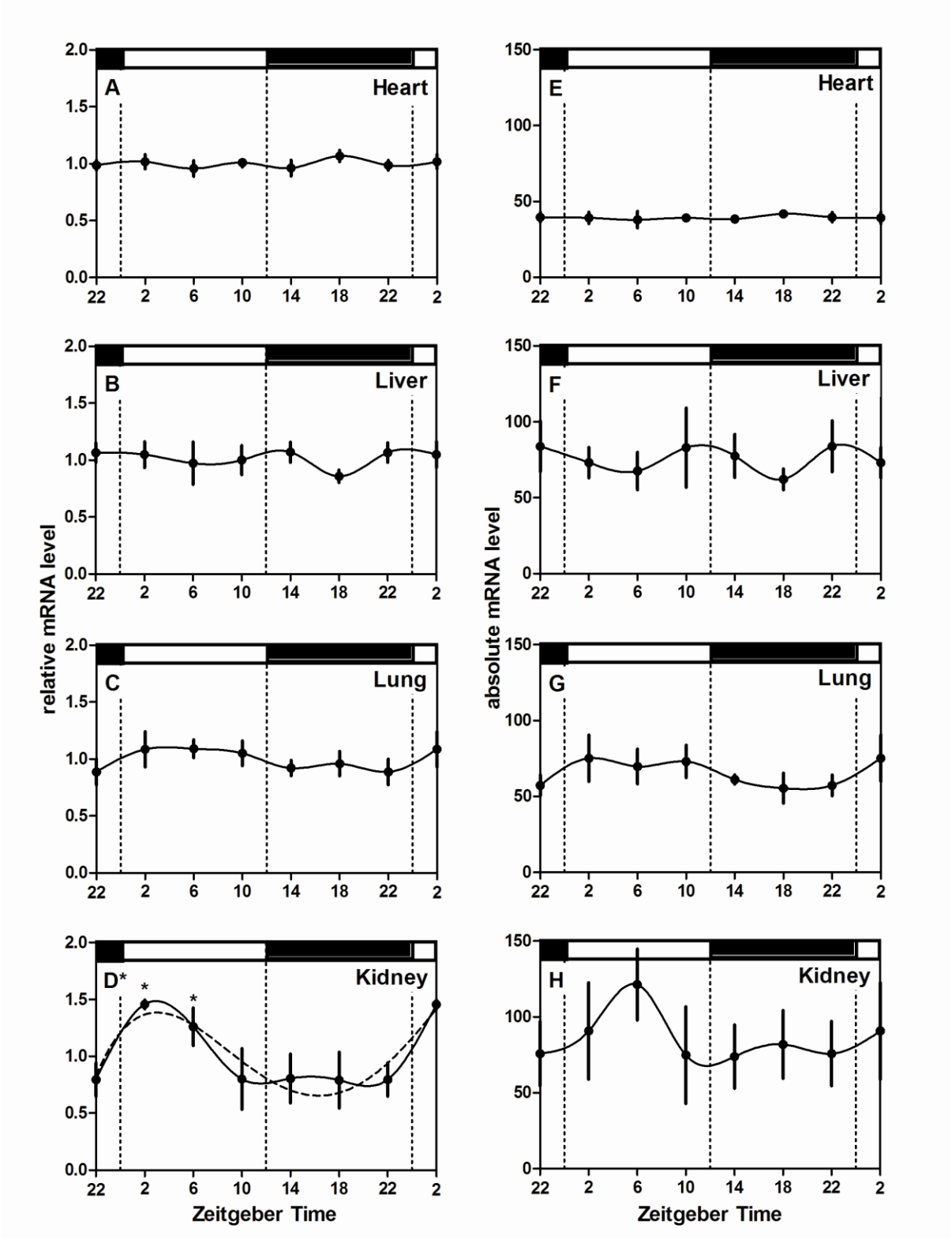
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